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Functional Survey for Heterologous Sugar Transport Proteins, Using *Saccharomyces cerevisiae* as a Host▽

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Molecular transport is a key process in cellular metabolism. This step is often limiting when using a nonnative carbon source, as exemplified by xylose catabolism in *Saccharomyces cerevisiae*. As a step toward addressing this limitation, this study seeks to characterize monosaccharide transport preference and efficiency. A group of 26 known and putative monosaccharide transport proteins was expressed in a recombinant *Saccharomyces cerevisiae* host unable to transport several monosaccharides. A growth-based assay was used to detect transport capacity across six different carbon sources (glucose, xylose, galactose, fructose, mannose, and ribose). A mixed glucose-and-xylose cofermentation was performed to determine substrate preference. These experiments identified 10 transporter proteins that function as transporters of one or more of these sugars. Most of these proteins exhibited broad substrate ranges, and glucose was preferred in all cases. The broadest transporters confer the highest growth rates and strongly prefer glucose. This study reports the first molecular characterization of the annotated XUT genes of *Scheffersomyces stipitis* and open reading frames from the yeasts *Yarrowia lipolytica* and *Debaryomyces hansenii*. Finally, a phylogenetic analysis demonstrates that transporter function clusters into three distinct groups. One particular group comprised of *D. hansenii XylHP* and *S. stipitis XUT1* and *XUT3* demonstrated moderate transport efficiency and higher xylose preferences.

Lignocellulosic biomass is an attractive industrial feedstock that can be converted into liquid transportation fuels and other small molecule bioproducts via microbial and fungal fermentations (6). However, this material is quite recalcitrant to enzymatic digestion and contains a significant fraction of pentose sugars (especially D-xylose and L-arabinose). These pentose sugars cannot be readily metabolized by nonrecombinant versions of common fermentative host organisms such as the bacterium *Zymomonas mobilis* or the yeast *Saccharomyces cerevisiae*. While native pentose-utilizing organisms exist, a lack of well-developed genetic tools and low product tolerances (13) limit their utility as hosts for industrial scale lignocellulosic conversion processes. As a result, a significant effort has focused on the metabolic engineering of pentose catabolic pathways in the yeast *S. cerevisiae* to enable xylose and arabinose fermentation (12, 18). Despite these efforts, the transport of these exogenous sugars is still limited and can often be the rate-limiting step in metabolism (8). It has been demonstrated that xylose transport in recombinant yeast is facilitated by native glucose transporters (14, 27, 37). The lack of a dedicated xylose transport system in recombinant *S. cerevisiae* thus limits the capacity for dual xylose and glucose fermentation as well as high xylose catabolic pathway flux (17). This limitation highlights the need to identify and/or engineer efficient, heterologous xylose transport proteins in yeast.

Initial work to enable xylose utilization in yeast focused on establishing the essential, heterologous catabolic pathways. To this end, several metabolic engineering strategies for enabling the recombinant fermentation of pentoses in the yeast *S. cerevisiae* have been investigated (1, 16, 23, 41). The vast majority of work has focused on the most abundant pentose sugar, D-xylose, through a combination of heterologous pathway engineering and native pentose phosphate pathway (PPP) optimization (12, 42). The introduction of one of two basic heterologous xylose catabolic pathways, an oxidoreductase pathway commonly found in fungi (20) and an isomerase pathway commonly found in bacteria (25), can confer growth on xylose as a sole carbon source. Both of these pathways convert xylose to the natively fermentable ketose sugar xylulose. However, the oxidoreductase pathway suffers from cofactor imbalance (18), whereas the isomerase pathway has proved difficult to actively express (21) and suffers from lower throughput. Further overexpression and complementation of the native PPP enzymes xylulokinase (XKS) (5), transaldolase (TAL) (19), and transketolase (TKT) (38) have improved xylose catabolic rates. Together, these metabolic engineering efforts have led to increased xylose catabolic flux and improved ethanol yields. However, independently of the pathway used, xylose flux in recombinant yeast has been shown to be limited by transport (8, 14, 26, 35). As a result, further pathway and metabolic engineering efforts aimed at improving intracellular pathways will only increase this limitation (42).

Heterologous xylose transporter expression to alleviate this limitation has been explored (14, 15, 28, 35). These studies suggest that heterologous transporters can improve *S. cerevisiae* xylose fermentation characteristics (27). However, only a few proteins have been experimentally identified for enabling xylose transport in *Saccharomyces cerevisiae*, and all of these have been shown to favor glucose over xylose in a mixed-sugar culture. Moreover, while these proteins show affinity toward two structurally different monosaccharides (glucose and xylose), no work has examined other monosaccharides as potential substrates. Such a characterization would expand our un-

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TABLE 1. Strains used in this study

Strain[a]	Description	Source or reference
Candida intermedia NCYC 2504	Wild type	NCYC
Debaryomyces hansenii CBS 767	Wild type	ATCC (ATCC 36239)
Escherichia coli 10-beta	*araD139* Δ(*ara-leu*)*7697 fhuA laxX74 galK* [ϕ80 Δ(*lacZ*)M15] *mcrA galU recA1 end A1 nupG rpsL* (Str^r) Δ(*mrr-hsdRMS-mcrBC*)	New England Biolabs
Escherichia coli K-12 MG1655	Sequenced *E. coli* strain	ATCC (ATCC 700926)
Scheffersomyces stipitis CBS 6054	Wild type	ATCC (ATCC 58785)
Yarrowia lipolytica	Wild type	ATCC (ATCC 8662)
S. cerevisiae BY4741	Standard laboratory yeast	EUROSCARF (accession no. Y00000)
S. cerevisiae EBY.VW4000	*MAT*α *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1 Mal2-8c SUC2 hxt17*Δ *hxt13*Δ::*loxP hxt15*Δ::*loxP hxt16*Δ::*loxP hxt14*Δ::*loxP hxt12*Δ::*loxP hxt9*Δ::*loxP hxt11*Δ::*loxP hxt10*Δ::*loxP hxt8*Δ::*loxP hxt514*::*loxP hxt2*Δ::*loxP hxt367*Δ::*loxP gal2*Δ *stl1*Δ::*loxP agt1*Δ::*loxP ydl247w*Δ::*loxP yjr160c*Δ::*loxP*	41
S. cerevisiae EY1	*S. cerevisiae* EBY.VW4000(p16T.*XYL1*)	This study
S. cerevisiae EY12	*S. cerevisiae* EBY.VW4000(p16T.*XYL1*)(p25G.*XYL2*)	This study
S. cerevisiae EY12.XX	*S. cerevisiae* EY12(p14T.XX)	This study

[a] This includes the strains from which genomic DNA was isolated, as well as the recombinant host EY12, which was used as a host for the experiments conducted in this study.

derstanding of molecular transporter function as well as suggest potentially useful classes of transport proteins for improving recombinant xylose utilization in yeast.

In this study, we pursue a functional survey and characterization of 23 heterologous and 3 native *S. cerevisiae* yeast proteins expressed in a recombinant xylose-utilizing *S. cerevisiae* host devoid of glucose and xylose transporters (40). These proteins represent both putative and known transporters capable of xylose transport spanning the organisms *Arabidopsis thaliana*, *Candida intermedia*, *Cryptococcus neoformans*, *Debaryomyces hansenii*, *Escherichia coli*, *Scheffersomyces stipitis* (formerly *Pichia stipitis* [24]), and *Yarrowia lipolytica*. We present growth-based assays using a variety of monosaccharides as sole carbon sources (glucose, xylose, galactose, fructose, mannose, and ribose) in an effort to characterize the substrate acceptance profiles of these transporters. Furthermore, we characterize the preference ratio of xylose to glucose using a competitive preference assay in order to measure the degree to which xylose transport is inhibited by glucose in a cofermentation. No prior study has evaluated putative transporters from several organisms, using a consistent strain background, in an effort to characterize carbon source profiles and preferences. Thus, these results present the largest-scale characterization of sugar transporter properties to date and suggest a path forward for improving xylose transport in recombinant *Saccharomyces cerevisiae* for biofuel applications.

MATERIALS AND METHODS

Strains and plasmids. The microbial strains and plasmids used in this study are listed in Table 1. *S. cerevisiae* EBY.VW4000 (40) was obtained as a gift from Eckhard Boles of the Institute of Molecular Biosciences, Goethe-Universität, Frankfurt, Germany. *D. hansenii* CBS 767, *E. coli* K-12 MG1655, *S. stipitis* CBS 6054, and *Y. lipolytica* ATCC 8662 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). *S. cerevisiae* BY4741 was obtained from EUROSCARF (Goethe-Universität, Frankfurt, Germany). *C. intermedia* NCYC 2504 was obtained from the National Collection of Yeast Cultures (NCYC; Colney, Norwich, United Kingdom). *Escherichia coli* 10-beta (New England Biolabs, Ipswich, MA) was routinely used for gene cloning. *E. coli* strains containing the Mumberg et al. yeast shuttle vectors for gene cloning (32) (ATCC 87669) were obtained from the ATCC. Vectors were isolated using a plasmid miniprep kit (Qiagen, Valencia, CA). A complete list of all vectors used in this study is given in Table 2.

Media and culture conditions. Yeast and bacterial strains were stored at −80°C in 15% glycerol. *E. coli* strains were grown in LB-Miller broth and supplemented with 50 μg/ml of ampicillin for plasmid propagation when necessary. *C. intermedia* and *S. stipitis* were cultivated at 30°C in YM broth (3 g/liter yeast extract, 3 g/liter malt extract, 5 g/liter Bacto peptone, 10 g/liter glucose). *D. hansenii*, *S. cerevisiae* BY4741, and *Y. lipolytica* were cultivated at 30°C in YP

TABLE 2. Plasmids constructed[a]

Plasmid	Description	Reference or source
p416-TEF	URA3, CEN6/ARSH4 origin, TEFp	33
p425-GPD	LEU2, 2μm origin, GPDp	33
p414-TEF	TRP, CEN6/ARSH4 origin, TEFp	33
p16T.X1	p416-TEF-SsXyl1	This study
p25G.X2	p425-GPD-SsXyl2	This study
p14T.02	p414-TEF-AtXYLL3	This study
p14T.03	p414-TEF-AtXYLL2	This study
p14T.04	p414-TEF-ScHXT3	This study
p14T.05	p414-TEF-CiGXF1	This study
p14T.06	p414-TEF-CiGXS1	This study
p14T.07	p414-TEF-DEHA0D02167	This study
p14T.08	p414-TEF-DEHA2B14278	This study
p14T.09	p414-TEF-DEHA2A14300	This study
p14T.10	p414-TEF-DEHA2F19140	This study
p14T.12	p414-TEF-DhXylHP	This study
p14T.13	p414-TEF-EcXylE	This study
p14T.14	p414-TEF-SsXUT1	This study
p14T.15	p414-TEF-SsXUT2	This study
p14T.16	p414-TEF-SsXUT3	This study
p14T.17	p414-TEF-SsXUT4	This study
p14T.18	p414-TEF-SsXUT5	This study
p14T.19	p414-TEF-SsXUT6	This study
p14T.20	p414-TEF-SsXUT7	This study
p14T.21	p414-TEF-YALI0B06391	This study
p14T.22	p414-TEF-YALI0B01342	This study
p14T.23	p414-TEF-YALI0F06776	This study
p14T.24	p414-TEF-YALI0C06424	This study
p14T.25	p414-TEF-YALI0C08943	This study
p14T.26	p414-TEF-CNBC3990	This study
p14T.27	p414-TEF-ScHXT7	This study
p14T.35	p414-TEF-ScHXT13	This study
p14T.36	p414-TEF-ScGAL2	This study

[a] The xylose metabolic genes *XYL1* and *XYL2* from *Scheffersomyces stipitis* were expressed in Mumberg et al. shuttle vectors (32), along with all known and putative transporter ORFs included in this study.

medium (10 g/liter yeast extract, 20 g/liter Bacto peptone) with 20 g/liter glucose. *S. cerevisiae* EBY.VW4000 was cultivated at 30°C in YP medium with 20 g/liter maltose (YPM) (40). All strains were cultivated with 225-rpm orbital shaking. To select transformants, yeast synthetic complete (YSC) medium composed of 6.7 g/liter yeast nitrogen base, 15 g/liter agar, and either complete supplement mixture (CSM)-Ura, CSM-Leu-Ura, or CSM-Leu-Trp-Ura (MP Biomedicals, Solon, OH) were added, depending on the required auxotrophic selection. The carbon source used for selection, propagation, and preculturing of EBY.VW4000-derived strains was 20 g/liter maltose. Growth characterization experiments used YSC medium with CSM-Leu-Trp-Ura and various carbon sources, detailed below.

Identifying known and putative transporter genes. The collection of 23 heterologous and 3 native *S. cerevisiae* transporters listed in Table 3 was chosen in order to characterize a more extensive carbon source profile and to compare novel open reading frames (ORFs) with native and literature-identified transporters. To this end, the *S. cerevisiae HXT7* and *GAL2* genes were included as positive xylose transport controls, and *S. cerevisiae HXT13* was included as a negative xylose transport control based on prior literature evidence (3, 14, 35, 37). The two *C. intermedia* genes, *GXF1* and *GXS1* (9, 35), were included to assay for additional monosaccharide substrates and to provide another benchmark from which to evaluate novel ORFs. Two ORFs from *Arabidopsis thaliana*, At5g59250 and At5g17010, were included to investigate a disagreement in the literature as to the ability of these transporters to confer improved xylose uptake characteristics (14, 15). The *Escherichia coli xylE* transporter was also included on the basis of its exclusive specificity for xylose (4), despite previous unsuccessful expression attempts in *S. cerevisiae* (14). The seven annotated *XUT* genes from *S. stipitis* were chosen in an attempt to identify the high-affinity xylose transporter(s) hypothesized to exist in this yeast. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (2) was then used to identify genes with a high degree of similarity to those transporters reported, yet those with over 90% homology were generally discarded. *C. intermedia GXS1*, *Trichoderma reesei xlt1*, and *S. stipitis XUT3* and *XUT4* served as the reference sequences for the BLAST search. ORFs from the sequenced yeasts *Y. lipolytica* and *D. hansenii* occurred frequently over multiple BLAST searches, which resulted in the inclusion of five ORFs from each organism in this study. *D. hansenii xylHP*, in addition to recovery by BLAST, has been mentioned as a potential xylose transporter (27). In addition, one gene from *C. neoformans* was included due to its homology to *S. stipitis XUT4*. Combined, these ORFs comprise the 23 heterologous transporter genes surveyed in this work. Since the *SUT1-3* genes of *S. stipitis* (22, 39) are so similar to each other and to *C. intermedia GXF1*, they were not included in this survey.

Cloning heterologous genes. PCR protocols utilizing Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA) were performed using standard protocols. The PCR primers used to amplify all genes as well as the restriction enzymes used in this study are listed in Table 3. Standard restriction enzyme cloning and bacterial transformations were performed according to the work by Sambrook and Russell (36). Yeast transformations were conducted according to the protocol described by Gietz and Schiestl (10). Genomic DNA was isolated from *C. intermedia*, *D. hansenii*, *E. coli*, *S. stipitis*, *S. cerevisiae*, and *Y. lipolytica* using the Wizard genomic DNA isolation kit (Promega, Madison, WI). *Arabidopsis thaliana* cDNA originally isolated from the CD4-30 library was provided as a gift from Alan Lloyd at The University of Texas at Austin. Since both *S. stipitis XUT4* and *C. neoformans* CNBC3990 possessed one or more introns, the ORFs were synthesized as an intron-free gene by Blue Heron Biotechnology (Bothell, WA). The native ORF was used; no yeast codon optimization was selected. All heterologous ORFs were cloned into the multiple cloning site of the Mumberg et al. plasmid p414-TEF (Table 2) and then sequence verified to ensure correct cloning. To create a xylose utilization pathway, *S. stipitis XYL1* was cloned into p416-TEF and *S. stipitis XYL2* was cloned into p425-GPD. All cloned genes were sequence confirmed after cloning and prior to yeast transformations. All primers were purchased from Integrated DNA Technologies (Coralville, IA), restriction enzymes were purchased from New England Biolabs (Ipswich, MA), and the remaining chemicals were purchased through Thermo Fisher Scientific (Waltham, MA).

Growth rate measurements. Growth rates of the transformed yeast strains were measured with a Bioscreen C system (Growth Curves USA, Piscataway, NJ) with biological triplicates using the wide-band filter (420 to 580 nm) recommended for optical density (OD) measurements. A 1-μl inoculum of fully grown culture was added to each well with 250 μl of YSC medium plus 20 g/liter of the carbon source under investigation (glucose, xylose, galactose, fructose, mannose, or ribose) as well as an additional condition that contained only 5 g/liter xylose. The experiment was run for 48 h with high continuous shaking and sampling every 10 min. Exponential growth rates were calculated using a solution algorithm written in MATLAB. Strains with average growth rates that were below that of the empty vector control are reported as *bc*, rates statistically equal to that of the empty vector control are labeled *ec*, and strains that demonstrated growth rates higher than that of the control but did not double in optical density in 48 h are reported as *dnd.* All other values are reported as calculated, and the growth rates that were most significant (with a Z test value that rounded to 1) were considered top performers.

Competitive fermentation assays. Glucose-and-xylose mixed-sugar fermentations were used to assay sugar preference by these transporters. These glucose-xylose cofermentations were performed at a high yeast optical density in biological duplicates. A 2-day preculture was used to inoculate 250 ml of YSC plus 20 g/liter maltose. Once this culture exceeded an optical density (OD) of 4 (~24 h), the cells were pelleted, resuspended, and inoculated into 40 ml YSC plus 20 g/liter glucose and 20 g/liter xylose at an initial approximate OD of 20. Sample time points were taken at 0, 1, 3, 4, 6, 8, 12, and 24 h. At each time point, a 500-μl sample was taken and pelleted for 2 min at full speed in a microcentrifuge. The supernatant was diluted 1:10, and the glucose and xylose concentrations were measured using the YSI Life Sciences bioanalyzer 7100 MBS. Initial glucose and xylose consumption rates were calculated based on the change in concentration of the sugars over time. The linear range of the glucose consumption curve was found, and the slope was calculated. Over the same time interval, a xylose consumption slope was also calculated. The ratio of xylose consumption rate to glucose consumption rate was then computed and converted to a mole ratio to give the X/G preference ratio. High values of the preference ratio would be interpreted as a xylose selective transporter, although no ORFs in this study exhibited such behavior. In addition to the preference ratio, the percentage of sugar transported was computed by subtracting the total sugar remaining after 24 h from the initial total amount of sugars and dividing that by the initial total amount of sugars. This metric provided an assessment of overall throughput of the transporter under study.

RESULTS

Cloning and selection of known and putative xylose transporters. A total of 26 transporters were evaluated in this study, consisting of 23 heterologous and 3 native *S. cerevisiae* transporter proteins, which comprise the largest survey of transporter ORFs for xylose transport function to date (Table 3). These transporters were selected based on homology searches and literature evidence of xylose transport capacity, as described above in Materials and Methods. This collection of transporters encompasses a wide array of phylogenetic diversity (Fig. 1). Once cloned into the shuttle vector p414-TEF, the transporters were transformed into a hexose transporter-null strain of yeast (EBY.VW4000) complemented with a *S. stipitis*-based oxidoreductase pathway (the genes *XYL1* and *XYL2* are expressed by plasmids). This strain, named EY12, was used throughout this study. This host background is unable to support substantial growth on glucose or xylose due to the transporter deletions, validated by our growth rate assays in which the strain was unable to double in OD in the 48-h duration of the experiment. Similar growth kinetics (lack of doubling within 100 h or more) have been observed for similarly constructed yeast strains (14). *S. cerevisiae* EY12 thus provides a suitable host for comparing specific functions of transporter proteins.

Growth phenotypes of recombinant strains using different carbon sources. In order to characterize sugar utilization profiles, the growth rates of *S. cerevisiae* EY12 strains harboring these 26 transporters were measured using glucose, xylose, low xylose (5 g/liter), galactose, fructose, mannose, or ribose as the sole carbon source in minimal media. With the exception of fructose, the host strain is unable to support growth under experimental conditions on any of these sugars without the aid of a heterologous transporter protein. Fructose supports low

TABLE 3. ORFs cloned[a]

Organism	Gene/locus tag	UniProt accession no.	Source or reference	Primer	RE(s) used	Sequence
S. stipitis	*XYL1*	P31867	24	EY037	XbaI	GCTCTAGAATGCCTTCTATTAAGTTGAACTCTGGTTAC
				EY038	ClaI	CCATCGATTTATTTCCTCTCTATAAAGCAACCTTCTTAG
	XYL2	P22144	24	EY103	BamH1-HF	GGCGCGGATCCATGACTGCTAACCCTTCCTTGGT
				EY104	XmaI	TCCCCCGGGGGATTACTCAGGGCCGTCAATGAGAC
A. thaliana	*At5g59250*	Q0WWW9	16	EY052	EcoRI	CGGAATTCATGGCTTTCGCTGTCTCGGT
				EY004	ClaI	CCATCGATTCACTTCAAGATTTTTGATTCAATTTCTTCC
	At5g17010	Q6AWX0	16	EY177	BamH1-HF	GGCGCGGATCCATGGCGCTTGATCCTGAGCA
				EY178	ClaI	CCGTATCCATCGATTTAGAGACATTTGGCTTCAATTTCCTCA
C. intermedia	*GXF1*	Q2MDH1	29	EY175	BamH1-HF	GGCGCGGATCCATGTCACAAGATTCGCATTCTCT
				EY176	ClaI	CCGTATCCATCGATTTAAACCTGTTCGTCGGTGGCC
	GXS1	Q2MEV7	29	EY083	BamH1-HF	CGGGATCCATGGGTTTGGAGGACAATAGAATGG
				EY082	ClaI	CCATCGATTTAAACAGAAGCTTCTTCAGACATAAATAGC
D. hansenii	*DEHA0D02167*	Q6BTD8	BLAST	EY163	XmaI	TATTCCCCCGGGATGGGTTTAGAAGATAATGCGCTTAT
				EY164	XhoI	CCGTGGCTCGAGTTAGACTGAAGTGGTTTCAATTTCAGT
	DEHA2B14278	Q6BW54	BLAST	EY087	BamH1-HF	CCATCGATCTACGAACTAGAGTCCTTGGTATCAAC
				EY086	ClaI	CGGGATCCGAGAGAAGGAGAAAAATAATACGAAATGTGG
	DEHA2A14300	B5RSN0	BLAST	EY181	EcoRI	GAGTGGCGGAATTCATGTTCAATAAAATCAGATTTGGTTTCTGCA
				EY182	ClaI	CCGTATCCATCGATCTACTTACTTATGCTATTTGATTCCACTTGTTC
	DEHA2F19140	B5RUJ3	BLAST	EY084	BamH1-HF	CGGGATCCTTGATGTCTTCGTTATTGACCAACA
				EY085	ClaI	CCATCGATTCAATTATTCGAGAGCAAAGTACGTTC
	xylHP	Q64L87	28	EY167	XmaI	TATTCCCCCGGGATGACTACTGCTGTTGGATTAGAAGATAATTCC
				EY168	XhoI	CCGTGGCTCGAGTTAATCAGAATAATGTGCTTCCGAAATATCTGTTG
E. coli	*xylE*	P0AGF4	15	EY051	BamH1-HF	CGGGATCCATGAATACCCAGTATAATTCCAGTTATATATTTTCG
				EY001	ClaI	CCATCGATTTACAGCGTAGCAGTTTGTTGTG
S. stipitis	*XUT1*	A3LY10	NCBI	EY073	BamH1-HF	CGGGATCCATGCACGGTGGTGGTGACGG
				EY072	ClaI	CCATCGATTTATTTTTCAACGTGGTAGACATCAGCCTTGC
	XUT2	A3GIE8	NCBI	EY061	BamH1-HF	CGGGATCCATGAAGTATTTTTCAAATCTGGAAATCAGGC
				EY032	ClaI	CCATCGATCACTCAACTTCAATATGCTCGATTATTG
	XUT3	A3GHU5	NCBI	EY169	XmaI	TATTCCCCCGGGATGAGAGAAGTTGGTATTCTTGATGTTGC
				EY170	XhoI	CCGTGGCTCGAGTTATTCTGACATTTCAATCGAGTTGCG
	XUT4	A3M0B9	NCBI; intron-free version synthesized by Blue Heron Biotechnology			
	XUT5	A3LY79	NCBI	EY068	EcoRI	CGGAATTCATGACGGAAAGAAGCATTGGACCTT
				EY069	ClaI	CCATCGATTTACTTCTTTGTATTAACAACAAAACCTTGTCTG
	XUT6	A3M0N4	NCBI	EY035	EcoRI	CGGAATTCATGTCCAGTGTTGAAAAAAGTGCTGAAA
				EY036	ClaI	CCATCGATTTAGCTGATGTTTTCGACATGCTCTAT
	XUT7	A3GHF2	NCBI	EY207	XmaI	TATTCCCCCGGGATGATATCATCGCTTTTGGTAGC
				EY174	ClaI	CCGTATCCATCGATCTAGAGTAATGTTCTTCTTGGAGACTCG
Y. lipolytica	*YALI0B06391*	Q6CFJ6	BLAST	EY059	EcoRI	CGGAATTCATGATTGGAAACGCTCAAATTAACCA
				EY027	ClaI	CCATCGATTTACAATTGAGAGGGAGGGGCG
	YALI0B01342	Q6CG30	BLAST	EY058	EcoRI	CGGAATTCATGTACAAGGTCCATAACCCCTACC
				EY026	ClaI	CCATCGATTTAGACATGCTCAGTTCCAGGATACT
	YALI0F06776	Q6C2L7	BLAST	EY023	BamH1-HF	CGGGATCCATGTTTTCGTTAACGGGCAAACCG
				EY024	ClaI	CCATCGATTTATACCGGAGGTTGAGGGAAGTC
	YALI0C06424	Q6CCU6	BLAST	EY171	XmaI	TATTCCCCCGGGATGGGACTCGCTAACATCATCAACC
				EY172	ClaI	CCGTATCCATCGATCTAGACAGACTCAATGTAGACGTGCTGTC
	YALI0C08943	Q6CCJ1	BLAST	EY210	XmaI	TATTCCCCCGGGATGGCCATTATTGTGGCTG
				EY211	ClaI	CGGTATCCATCGATCTAATCCGAATCAAATCCAGAAT

C. neoformans	*CNBC3990*	Q55VT8	BLAST	Introns, Synthesized by Blue Heron Biotechnology		
S. cerevisiae	*GAL2*	P13181	3, 38	EY220	EcoRI	TATTCCGAATTCATGGCAGTTGAGGAGAA
				EY221	ClaI	CGGTATCCATCGATTTATTCTAGCATGGCCTTG
	HXT4	P32466	15, 36	EY194	XmaI	TATTCCCCCGGGATGAATTCAACTCCAGATTTAATATCTCC
				EY208	ClaI	CGGTATCCATCGATTTATTTCTTGCCGAACATTTTCTT
	HXT7	P39004	15, 36	EY159	BamHI-HF	GGCGCGGATCCATGTCACAAGACGCTGCTATTGC
				EY180	ClaI	CCGTATCCATCGATTTATTTGGTGCTGAACATTCTCTTGTACAATGG
	HXT13	P39924	15, 36	EY196	XmaI	TATTCCCCCGGGATGTCTAGTGCGCAATCCTC
				EY209	ClaI	CGGTATCCATCGATTCAATCAGAATTCTTTGAGAACTTC

[a] Many genes were cloned using PCR and restriction enzyme (RE) cloning. The complete list is given here, including the restriction enzymes and primers used.

FIG. 1. Phylogenetic analysis of transporter proteins used in this study. Cladogram of protein sequences included in this study assembled by ClustalW and visualized in TreeView X. The functional ORFs show a great deal of clustering. Group A consists of ORFs with low growth rates, and only *C. intermedia GXS1* confers growth on xylose. Group B consists of nonspecific but efficient transporters. Members of group C confer xylose growth and exhibit moderate to high xylose-to-glucose preference ratios yet still prefer glucose. Prefixes: At, *A. thaliana*; Ec, *E. coli*, Ci, *C. intermedia*; Dh, *D. hansenii*; Yl, *Y. lipolytica*; Sc, *S. cerevisiae*; Ss, *S. stipitis*; Cn, *C. neoformans*. X, xylose; G, glucose.

growth rates in the control strain (an OD doubling occurred within 48 h). Of the 26 transporters evaluated, 10 ORFs conferred statistically significant growth phenotypes on one or more of these tested carbon sources. Table 4 depicts the carbon source-associated growth rates for these 10 transporters compared with that for an empty vector control. The remaining 16 transporters did not enable growth at a rate that was significantly above the control (denoted *bc* or *ec*) or did not double in optical density in 48 h (*dnd*) (Table 5). It is important to note that the EY12 strain used to express these transporters is a highly modified strain of yeast (with over 20 gene knockouts and three plasmids) and thus is not expected to exhibit absolute growth rates that match those of industrial or unmodified strains of yeast. However, the qualitative trends and rank order analysis presented here provide a means of comparing the performance of isolated heterologous transporters.

This growth rate assay enables several observations about the behavior of molecular transport proteins. First, our results generally show both broad and narrow specificities for the carbon sources assayed. Of the 10 ORFs conferring growth phenotypes in *S. cerevisiae* EY12, all of them restored growth on glucose, whereas only 7 also conferred growth on xylose. These 7 transporters all conferred growth on the other hexoses

TABLE 4. Growth rates conferred by transporter expression in *S. cerevisiae* EY12[a]

Condition or source organism	Gene/locus tag	Growth rate (μ [per h] $\pm$ σ) on indicated carbon source						
		20 g/liter glucose	Xylose		20 g/liter galactose	20 g/liter fructose	20 g/liter mannose	20 g/liter ribose
			20 g/liter	5 g/liter				
Media only		0.000 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Empty vector		*dnd*	*dnd*	*dnd*	*dnd*	0.047 ± 0.004	*dnd*	*dnd*
S. cerevisiae	*HXT7*	**0.127 ± 0.008**	**0.090 ± 0.002**	0.076 ± 0.001	**0.095 ± 0.001**	**0.165 ± 0.005**	**0.151 ± 0.014**	*bc*
	HXT13	0.060 ± 0.009	*ec*	*ec*	*ec*	*dnd*	0.085 ± 0.003	*bc*
	GAL2	0.058 ± 0.008	**0.112 ± 0.02**	**0.108 ± 0.008**	**0.129 ± 0.000**	**0.131 ± 0.006**	**0.173 ± 0.000**	**0.093 ± 0.005**
C. intermedia	*GXF1*	**0.172 ± 0.006**	0.070 ± 0.003	**0.085 ± 0.004**	0.081 ± 0.012	**0.194 ± 0.006**	**0.182 ± 0.013**	*bc*
	GXS1	0.051 ± 0.009	0.049 ± 0.004	0.078 ± 0.011	0.065 ± 0.003	0.070 ± 0.002	0.076 ± 0.008	0.074 ± 0.002
D. hansenii	*DEHA0D02167*	0.045 ± 0.007	*dnd*	*ec*	0.091 ± 0.003	0.067 ± 0.001	0.081 ± 0.002	*ec*
	XylHP	0.073 ± 0.005	0.065 ± 0.008	0.080 ± 0.007	**0.100 ± 0.007**	0.072 ± 0.002	0.101 ± 0.021	*ec*
S. stipitis	*XUT1*	0.063 ± 0.009	0.050 ± 0.001	*ec*	0.085 ± 0.003	0.061 ± 0.011	0.066 ± 0.002	*ec*
	XUT3	0.058 ± 0.007	0.057 ± 0.005	0.069 ± 0.004	**0.093 ± 0.003**	0.053 ± 0.005	0.064 ± 0.006	*ec*
Y. lipolytica	*YALI0C06424*	0.043 ± 0.009	*dnd*	*ec*	*ec*	*bc*	*bc*	*ec*

[a] Here the growth rate (μ) is reported for the 10 genes that conferred growth on any of the carbon sources assayed. Standard deviations are reported, and the genes with significant *Z* test scores for each carbon source are shown in boldface. The top performer in each category is shaded. Generally, the transporters assayed show a trade-off between efficiency and specificity. There are three abbreviations used to indicate a slow-growth phenotype. The measured growth rate was either below that of the control (*bc*), indicating detrimental effects on the host, equal to that of the control (*ec*), showing performance statistically similar to that of the empty vector control, or did not produce a doubling in optical density in 48 h (*dnd*).

assayed. The other 3 transporters showed various specificities for hexoses. *Y. lipolytica YALI0C06424* conferred growth on glucose only, *S. cerevisiae HXT13* conferred growth on glucose and mannose, and *D. hansenii DEHA0D02167* was specific for hexoses. Growth on ribose was conferred only by *S. cerevisiae GAL2* and *C. intermedia GXS1*, making this phenotype the rarest among the ORFs assayed. No strain assayed was found to grow solely on xylose; only strains that grew solely on glucose were observed.

Second, these data show a clear relationship between substrate specificity and transporter efficiency. Highly specific transporters conferred the lowest growth rates for those carbon sources on which they grew. *S. cerevisiae GAL2*, *C. intermedia GXF1*, and *S. cerevisiae HXT7* were the least specific transporters and consistently conferred the highest growth rates. An exception to this trend is the broad transporter *C. intermedia GXS1*, which conferred modest to low growth on most carbon sources. This phenomenon may be due to the H^+ symport mechanism of Gxs1p (28), which would reduce its efficiency compared to facilitated diffusion transport proteins when protons are scarce, unlike with xylose. However, *C. intermedia GXS1* expression provided a 60% increase in the conferred growth rate when grown on 5 g/liter xylose compared to that when grown on 20 g/liter xylose. This improvement is a nearly 3-fold percent increase compared to those of *C. intermedia GXF1*, *D. hansenii xylHP*, and *S. stipitis XUT3*. Again, this improvement may be due to the proton symport mechanism of Gxs1p. In contrast, the native *S. cerevisiae* proteins all demonstrated decreased growth rates in reduced xylose concentrations.

Third, this study enables rank order analysis of the magnitude of growth rates for a given carbon source but not across carbon sources due to factors such as differing metabolic pathways. *C. intermedia GXF1* conferred the highest growth rate on glucose, fructose, and mannose in addition to its high growth rate on 5 g/liter xylose. *S. cerevisiae GAL2* was the most efficient transporter for xylose, galactose, and ribose in addition to high growth rates conferred when grown on fructose and mannose. Including the results obtained by this study, *S. cerevisiae GAL2* has now been found to facilitate the transport of the following three different pentoses: xylose (14) (this study), ribose (this study), and L-arabinose (3). *S. cerevisiae HXT7* also conferred high growth rates on all carbon sources except ribose and 5 g/liter xylose. *D. hansenii xylHP* and *S. stipitis XUT3* expression enabled high growth rates on galactose.

Fourth, these experiments demonstrate that a high fraction of proteins putatively annotated as xylose transporters do not confer growth on any monosaccharide carbon source when

TABLE 5. ORFs that did not confer growth in *S. cerevisiae* EY12[a]

Organism	Gene/locus tag/promoter
A. thaliana	*At5g59250*
	At5g17010
C. neoformans	*CNBC3990*
D. hansenii	*DEHA2A14300p*
	DEHA2B14278p
	DEHA2F19140p
E. coli	*xylE*
S. stipitis	*XUT2*
	XUT4
	XUT5
	XUT6
	XUT7
Y. lipolytica	*YALI0B06391p*
	YALI0B01342p
	YALI0C08943p
	YALI0F06776p

[a] This list of 16 transporters exhibited *ec*, *bc*, or *dnd* growth phenotypes for all carbon sources used in this study.

heterologously expressed in recombinant *S. cerevisiae*. These 16 transporters are listed in Table 5. Most of the *S. stipitis XUT* family and *E. coli* and *A. thaliana* ORFs are included. The results for the plant and bacterial genes support the findings of Hamacher et al. (14). As for the *S. stipitis* transporters, it is not clear from our study why so many of them conferred no significant growth phenotype.

Future work is required to determine whether these proteins are not actually transporters, may transport other carbon sources not tested, or are not suited for heterologous expression in recombinant *S. cerevisiae* due to folding or membrane compatibility differences. There is also the possibility that one or more of these proteins acts as a membrane sensor protein that does not transfer substrates across the cellular membrane, like the *RGT2* glucose sensor (7). Nevertheless, no observations of significant growth rates for a majority of the transporters assayed indicates a high failure rate for BLAST-identified ORFs. Also, the low number of ORFs able to confer growth on xylose may indicate that specific xylose transporters are structurally different from the reference genes used for the BLAST algorithm or are inefficient when expressed individually.

Collectively, these results upgrade our understanding of monosaccharide transport in several organisms. To our knowledge, this study presents the first experimental characterization of individual monosaccharide transporters from the yeasts *D. hansenii* and *Y. lipolytica* and of the *S. stipitis XUT* family. Within these organisms, it is evident that *S. stipitis XUT1* and *XUT3* and *D. hansenii xylHP* enable the transport of hexoses and xylose, while *D. hansenii DEHA0D02167* and *Y. lipolytica YALI0C06424* appear to be hexose transporters. These results also present the first characterization of *S. cerevisiae HXT7*, *HXT13*, and *GAL2* and *C. intermedia GXF1* and *GXS1* on hexose and pentose sugars other than glucose and xylose.

Competitive preference of xylose in the presence of glucose. Most industrial applications with engineered yeasts will be in an environment where multiple sugars are present. Thus, it is also important to evaluate the relative preference exhibited by transporter proteins for sugars as a means of qualitatively assessing Michaelis constant (K_m) values for these transporters. It is inadvisable to compare growth rates across carbon sources in Table 4 to understand preference, since competitive inhibition may interfere with the ability to consume the sugar at the rates occurring in the sole carbon source growth assay. Thus, to address this issue, a competitive fermentation assay was conducted to test substrate preference in a more direct manner. This glucose-xylose cofermentation experiment was conducted with a high-cell-density culture of yeast cells with 20 g/liter glucose and 20 g/liter xylose present at the same time. The extracellular concentration of these sugars was monitored over time, and the consumption rate of each sugar was measured. The ratio of these consumption rates was computed to give the xylose to glucose preference ratio (termed the X/G preference ratio). These preference ratios are provided in Table 6. All of the transporters tested had low X/G preference ratios; the top performer imported only one molecule of xylose per two molecules of glucose. Thus, this experiment demonstrates that these transporter proteins possess a stronger preference for glucose over xylose. In addition, the percentage of total sugar consumed was calculated by determining the total amount of the 40 g/liter of sugars that had been consumed after 24 h of culturing and dividing that by 40 g/liter of sugars (Table 6). The percent total sugar consumed is a facile measure of collective transporter efficiency.

TABLE 6. Xylose-to-glucose preference ratios and percent total sugar consumed during the competitive preference assay[a]

Organism	Gene	X/G preference ratio	% sugars consumed
S. cerevisiae	*HXT7*	0.11	75
	GAL2	0.09	68
C. intermedia	*GXF1*	0.10	78
	GXS1	0.51	14
D. hansenii	*xylHP*	0.17	58
S. stipitis	*XUT1*	0.69	16
	XUT3	0.11	17

[a] A cofermentation assay was conducted to determine transporter preference. The X/G preference ratio is calculated by first measuring the molar consumption rates of each sugar over the same time period from a stationary-phase yeast culture and then dividing the xylose molar consumption rate by the glucose molar consumption rate. This metric approximates how many xylose molecules per glucose molecule are imported. As a measure of transporter throughput, the total sugar consumption over 24 h was measured and taken as a mass percentage of total sugars. Several cultures exhibit diauxic sugar consumption, in which glucose is consumed first, resulting in low X/G preference ratios and high percent sugars consumed.

These results demonstrate that glucose preference and transport efficiency are linked. In particular, the most efficient transporters typically exhibited the lowest preference ratios, whereas the opposite was true for the least efficient transporters. *S. stipitis XUT1* demonstrated the highest preference for xylose over glucose among the ORFs studied. However, this transporter was capable of importing only 16% of the sugars present in 24 h. The transporter with the second highest preference ratio, *C. intermedia GXS1*, also transported only 14% of total sugars. Moreover, the native transporters *S. cerevisiae GAL2* and *S. cerevisiae HXT7* exhibit a preference ratio below 0.1, indicating that these transporters mediate glucose uptake almost to the exclusion of xylose. Nevertheless, these transporters are quite efficient at total utilization, as over 65% of the sugars present were transported in 24 h. Therefore, the expression of these transporters creates a strong diauxic growth phenotype. Likewise, *C. intermedia GXF1* and *D. hansenii xylHP* had low preference ratios (0.10 and 0.17, respectively) yet consumed 78% and 58% of the total sugars, respectively. Finally, *S. stipitis XUT3* had low performance with both metrics. In all of these cases, glucose preference is directly linked with transport efficiency.

DISCUSSION

This study provides the first large-scale analysis of substrate specificity and preference across a varied group of putative and known transporter proteins. By studying 26 transporters from seven different organisms, we evaluated a diverse collection of transporters and identified 10 that function as monosaccharide transporters in the absence of the native hexose transport system of *S. cerevisiae*. Our results reveal that efficiency trends inversely with specificity and directly with glucose preference. These results are expected when considering the diauxic growth phenomena observed for a large number of microbes, including *S. stipitis* (30), and that broad substrate efficiency is evolutionarily advantageous, since naturally available carbon

sources are somewhat unpredictable. Therefore, expression of an efficient, broad transporter allows a great deal of flexibility without requiring the induction and expression of new transporters for different carbon sources.

The most specific transporters in this study (*S. cerevisiae HXT13* and *Y. lipolytica YALI0C06424*) exhibited low overall efficiency. Therefore, it is presumed that loss of transporter efficiency is the fitness trade-off associated with improved specificity. This trade-off does not bode well for discovering an efficient, xylose-specific yeast transporter in organisms that have evolutionarily been exposed to a broad range of carbon sources. While xylose-preferring transporters may exist in yeast, they are likely to exhibit low net transport rates and efficiencies based on the evidence described here. Exclusive xylose transporters are also likely be rare, since glucose exclusivity is shown to be rare in this study. Yet, it is known that the *E. coli xylE* transporter functions as an exclusive xylose transporter (4), so these transporters exist in bacteria. The unsuccessful expression of *xylE* in *S. cerevisiae* (14) (this study) could be due to membrane incompatibility, expression, and folding difficulties experienced with bacterial proteins. A similar problem exists when the bacterial xylose isomerase pathway is imported (31), which suggests a protein engineering approach could enable the use of this transporter in yeast.

Collectively, this study provides a sugar preference profiling of native and heterologous transporters. Sixteen of these proteins conferred no growth on any of the carbon sources assayed. Host incompatibility may partially explain these observations, although there are many possible explanations. Nevertheless, there are few explanations for observed functionality other than that the transporter is expressed and naturally accepts the specific carbon source. Therefore, this study reconfirms that *S. cerevisiae HXT7* transports glucose and xylose (14, 35, 37) and provides evidence that the transporter accepts additional hexoses. *S. cerevisiae HXT13* is reconfirmed as being able to transport glucose yet not xylose (14), and evidence here demonstrates an affinity for mannose. This study also supports the previous research involving *S. cerevisiae GAL2* (14, 37) and, further, finds that the transporter accepts all carbon sources studied, in addition to arabinose (3). *S. cerevisiae GAL2* thus encodes a very broad transporter.

Of the heterologous transport proteins, the two *C. intermedia* transporters are probably the most extensively studied (28, 29, 34). Previous reports focus on only the glucose and xylose transport capabilities of these proteins. The findings shown here indicate that these ORFs encode broad hexose transporters that have additional affinity for pentoses—xylose for *GXF1* and both xylose and ribose for *GXS1*. This study also shows that *D. hansenii xylHP*, previously indicated as a potential candidate for heterologous expression (27), is also a hexose transporter with affinity for xylose. The only other functional ORF from *D. hansenii*, *DEHA0D02167*, appears to encode a hexose transporter only. While previous studies were unable to identify functional transporters from *S. stipitis* (14), this study reports that *S. stipitis XUT1* and *XUT3* encode hexose transporters with affinity for xylose. Previously uninvestigated *Y. lipolytica YALI0C06424* is an exclusive glucose transporter; therefore, we propose that the annotation of this ORF be reclassified as a glucose transporter and be renamed as a HXT protein. Most of these characterizations demonstrate previously unknown substrates for these transporter proteins. This includes the first experimental validation for the *XUT* family. All other ORFs assayed have unknown functions, as they did not support growth on any of the carbon sources used in this study.

Of all the carbon sources assayed, ribose was seen as a substrate for only two proteins, *S. cerevisiae GAL2* and *C. intermedia GXS1*. It remains to be explained why these hexose transporters should have such affinity for pentoses. Ribose transport activity may be a concomitant result of other adaptations rather than an advantage, since free ribose in nature is rare. Also, the rarity of ribose acceptance implies that certain limits to broad substrate specificity exist. The permeases investigated are not merely open pores but engage in some type of protein-mediated substrate recognition. The ubiquity of glucose transport among these proteins illustrates a potential evolutionary path taken among these proteins. Moreover, these findings agree with what is already known about the metabolism of most microorganisms—hexoses are preferred carbon sources. These data also provide evidence that ribose transport across the cell membrane is almost unnecessary for most organisms or is carried out by a different class of proteins.

Our results also show that in a cofermentation, glucose transport is preferred over xylose; all ORFs investigated imported, at the most, one molecule of xylose for every two of glucose. While this phenomenon was observed only within the limited search space of this study, it may be the result of natural selection to produce sugar transporters that prefer hexoses. Even so, it remains possible that xylose fermenters such as *S. stipitis* and *D. hansenii* have dedicated xylose transport systems similar in scope to the hexose transport systems of *S. cerevisiae* based on their respective whole-cell transport phenotypes (11, 33). However, even *S. stipitis* demonstrates a diauxic growth phenotype in glucose-xylose cofermentation (11). Therefore, if xylose-preferring transport systems do exist in organisms such as *S. stipitis*, they are likely to have very low throughput and be quite distinct in sequence space compared to those of the glucose transporters.

As a final analysis to link protein sequences and observed phenotype, a phylogenetic tree was created based on the multiple protein sequence alignment of these 26 transporters generated by ClustalW (Fig. 1). As is evident from this figure, all of the transporters that confer growth on xylose cluster into three distinct functional groups. The first group (denoted group A on the tree) is characterized by the permissive proton symporter *C. intermedia GXS1*, which also demonstrates one of the highest xylose-to-glucose preference ratios. However, the other members of this group are specific for hexoses. Group B is composed of *C. intermedia GXF1* and several native *S. cerevisiae* transporters, which have very efficient transport characteristics yet very low preference ratios. Group C is composed of transporters from *S. stipitis* and *D. hansenii xylHP*, which all demonstrate moderate efficiencies and high preference ratios. Therefore, group C presents favorable candidates for further bioprospecting.

In conclusion, this survey revealed a subset of heterologous transporters that, when expressed in a hexose transporter-null strain of *S. cerevisiae*, permit growth on glucose and on xylose as well as on other hexose and pentose sugars. These transporters cluster into three well-defined groups, one of which is

worthy of further investigation for lignocellulosic biomass fermentation. This study presents the first molecular characterization of ORFs from several organisms of industrial interest across multiple carbon sources. As a result, novel ORFs from the yeasts *S. stipitis* and *D. hansenii* that are able to confer growth on xylose in recombinant *S. cerevisiae* were identified. In addition, we have demonstrated that transporters in nature exhibit a trade-off between specificity and efficiency. As a result, the solution to xylose transport limitations in yeast may require the development of new genetic approaches.

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